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THE EVALUATION OF SENSITIVITY AND SPECIFICITY OF NEW DEVELOPED ^{99M}Tc-OFLOXACIN RADIOTRACER TO DISCRIMINATE INFECTION INDUCED BY *STAPHYLOCOCCUS AUREUS* AND STERILE INFLAMMATION INDUCED BY CARRAGEENAN IN RAT'S FOOT

Alireza Doroudi [*1], Ali Kiasat [2], Maryam Saadi [1], Seyyed Mostafa Saadati [2], Faramarz Ahmadi [2], Behrooz Etesami [2], Mohammad Javad Khodayar [1] and Mostafa Erfani [3]

School of Pharmacy [1], Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.
Nuclear Medicine Department [2], Golestan General Hospital Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.
Radiation Application Research School [3], Nuclear Science and Technology Research Institute (NSTRI), Tehran, Iran.

Keywords:

Carrageenan, Infection-seeking, Ofloxacin, Radioisotope imaging, Sterile Inflammation, ^{99m}Tc-Ofloxacin

Correspondence to Author:
Dr Alireza Doroudi

School of Pharmacy,
Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

E-mail: Doroudi-a@ajums.ac.ir

ABSTRACT: The early identification of infection from sterile inflammation is still challenging step in nuclear medicine. Therefore, several radiopharmaceutical agents have been examined to find the solution of this dilemma in clinical practice. Recently ^{99m}Tc-Ofloxacin has been developed as an infection-seeking radiotracer. This investigation was launched to examine the sensitivity and selectivity of new developed radiotracer to distinct septic from aseptic lesions. The freeze-dried new developed kits were reconstituted by the 740 MBq (20 mCi) freshly $^{99m}TcO_4$. The radiochemical purity of ^{99m}Tc-Ofloxacin was undertaken by thin layer chromatography (TLC) and Radio high performance liquid chromatography (Radio-HPLC) analysis. Twenty adult, male NMRI rats were randomly divide into two groups equally. Infection was induced by *S. aureus* and sterile inflammation created by Carrageenan test. Then radioisotope studies have been performed after intravenous administration of radiotracer into the contra lateral tail vein in all studies. TLC and Radio-HPLC investigations indicated that the infection-seeking radiotracer could be prepared with appropriate yield. All lesions were visualized by scintigraphy imaging. The quality of images was good and did not change with over time. The sensitivity, specificity and positive predictive value of imaging with new developed radiotracer were 100, 50 and 50 % respectively. In spite of high sensitivity of radionuclide imaging with ^{99m}Tc-Ofloxacin to localize induced lesions, it could not demonstrate to distinct infection from sterile inflammation foci. The other medical modalities must be considered for intelligent interpretation of images if the new developed radiotracer will be introduced as an infection-seeking radiopharmaceutical in clinical practice.

INTRODUCTION: The distinct identification of infection from sterile inflammation lesions has prominent role on the therapeutic management of patients suspected of having such disorder entities. Infection is the result of invasion and localize of microorganism pathogens in the body, whereas inflammation is the natural response of the immune system against any other type of disorder or injury. Therefore, infection without inflammation or inflammation without infection may be happened. It depends upon the cause of the injury or disease. Different imaging techniques have been examined to discriminate between infection and sterile inflammation foci. Plain radiography is commonly used as a first attempt to evaluate the lesions created by infection or sterile inflammation.

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The other available imaging techniques, like ultrasonography, computerized tomography (CT) and magnetic resonance imaging (MRI) are highly sensitive, but these modalities have lack of selectivity for distinction of septic from aseptic foci, especially in early phase of disease when anatomic structures have not been considerably distorted [1, 2]. These techniques are dependent on morphologic abnormality changes that may not be present in the early stage of patient's disease. Radionuclide imaging can be considered as a part of the diagnosis procedures for detection of infection from sterile inflammation lesions [3]. This modality can be very helpful, because the whole body is investigated and providing information on physiologic changes of organs. Radioisotope imaging by different radiopharmaceuticals have been suggested in order to detect infection from sterile inflammation lesions.

An ideal radiopharmaceutical agent has the following characteristics to distinct infection or inflammation lesions. It has high sensitive to infection or inflammation site. It should discriminate infection from inflammation. The toxicity and immunogenic reactions have not been observed after administration of radiotracer agent to the patient. It has a rapid clearance from the blood and no gastrointestinal uptake. In addition to the aforementioned factors, the reconstitution of cold kit is easy and low cost at the nuclear medicine departments. Since no currently available radiopharmaceutical agents is not ideal in this regard. Each radiopharmaceutical has especial advantage and disadvantage characteristics [4-8].

Recently, the labeled ofloxacin with technetium radionuclide (^{99m}Tc-Ofloxacin) has been examined as an infection-seeking agent to detect induced septic foci in mice [9, 10]. Several investigational radiopharmaceutical agents have been demonstrated the potential capability to discriminate infection and sterile inflammation loci in the preliminary and pre clinic studies. But the most of them were not specific to discriminate septic or aseptic foci in clinical practice. It is highly desirable to establish a reliable experimental assay in preliminary phase in order to determine the specificity of any radiopharmaceutical agents which are investigated as an infection-seeking agent.

Carrageenan test is commonly used as an experimental assay for inducing artificial sterile inflammation lesion in the animals. This in-vivo experimental model has been frequently used to assess the anti-inflammatory effect of natural or synthetic compounds [11, 12]. The main of this approach is to evaluate the sensitivity and selectivity of imaging with new developed ^{99m}Tc-Ofloxacin radiotracer to identify infection induced by *Staphylococcus aureus* (*S. aureus*) and sterile inflammation lesions in rat's foot.

MATERIALS AND METHOD: All chemical materials have been purchased from Merck and Sigma. The chemicals and solvents were the highest purity and analytical grade and used without further purification. The new developed ofloxacin kits and ^{99}Mo/^{99m}Tc generators have been provided by Radioisotope Division of Atomic Energy Organization of Iran (AEOI). Technetium 99m as sodium pertechnetate was obtained from an in-house ^{99}Mo/^{99m}Tc generator using 0.9 % saline. The rats with average weight 130±15g were obtained from research center and experimental animal house of Ahvaz Jundishapur University of Medical Sciences.

***S. aureus* sample preparation:** Wild *S. aureus* samples were obtained from the patients admitted to the infection ward of Razi general public hospital in Ahvaz, Khuzestan Iran. The sample wound swabs were taken from the patients. The specimens were transported in sterile, leak-proof container to the laboratory of microbiology department. The isolates were inoculated on blood agar and incubated for 24 h at 35 ºC aerobically. Gram- positive cocci bacteria have been selected that occurring in pairs, short chains or clusters and were Catalase-positive. The surface of morphological identical isolated colonies was touched by a sterile-tip applicator. The applicator was immersed into a tube containing Mueller Hinton broth. The broth was incubated at 35 ºC, and then the turbidity was adjusted to a number 0.5 McFarland standard. A sterile cotton swab on a wooden stick was dipped into the broth. Excess inoculums were removed rotating the swab against of the tube above the fluid level wall. The Muller-Hinton agar plates were streaked in three dimensions. The plates were inoculated overnight at 35 ºC.

The turbidity was adjusted to a number o.5 McFarland (each milliliter of 0.5 McFarland contains 1.5×10^8 microorganisms). Half milliliter of inoculums has been injected to each rat's foot. To make sure about the survival of bacteria, 0.1 milliliter of the above mentioned inoculums was inoculated on blood agar. The experiment has been repeated three times.

Animal study: This study was approved by the ethics committee of Ahvaz Jundishapur University of Medical Sciences. All the ethics issues were considered based on the Ahvaz University of Medical Sciences Protocols (AUMP) on animal experiments. Therefore, all animal experiments were carried out in accordance with the local AUMP guideline. A total number of 20 adult, male NMRI were acclimated to the conditions for one week before the experiment. The animals were kept in individually wire-bottom stainless steel cages in an air-conditioned room at 24±1ºC with a 12 h light-dark cycle and were fed with standard pellet diet and had free access to water. The animals were randomly divided into two groups equally. Septic lesions were induced by S aureus in rat's foot in one group. Aseptic foci were created by Carrageenan solution in rat's foot in the other group.

Induced septic foci: The rats were briefly anesthetized by diethyl ether. *S. aureus* suspension was injected in the thigh muscle of rats in order to create septic foci. The injured area was irrigated with normal saline. The animals were return back to their cages. Radioisotope investigations have been performed 48 h after inoculation of bacteria suspension.

Induced aseptic foci: Half milliliter of 3% Carrageenan solution in saline was injected in the right thigh muscle of anesthetized rats on the experiment day for radioisotope analysis. The visible redness and pronounced swelling was created by Carrageenan 2h post injection. Maximum effect of Carrageenan has been observed between 2 to 4 h after injections and persisted for more than 12 h.

Preparation of ^{99m}Tc-Ofloxacin and quality control: Each new developed ofloxacin vial contained 1.5 mg ligand (ofloxacin antibiotic) and 75µg $SnCl_2$. $2H_2O$. The 740 MBq (20 mCi) freshly eluted $^{99m}TcO_4$ in 2 ml saline was added to the evacuated vial and incubated for 30 min at room temperature in lead shielded container. The radiochemical purity of ^{99m}Tc-Ofloxacin was determined by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) system. TLC plates (silica gel) were developed in methanol: water: ammonium hydroxide (2: 5: 1 v/v) as well as in acetone. In the first solvent, free $^{99m}TcO_4^-$ and ^{99m}Tc-Ofloxacin move with solvent front with R_f = 1 and the reduced technetium 99mTcO2 remain at the point of application (R_f = 0). In the second solvent, $^{99m}TcO_4^-$ move with solvent front with R_f = 1 and the other species remain at the point of application.

Then the strips were cut ⅓ lower and ⅔ upper pieces Each piece was counted for 2 minutes under a single head camera equipped with low energy all-propose collimator using an energy peak centered a140keV with NaI (Tl) detector. For radiochemical analysis by HPLC 20 µL aliquots were taken out at 1h post reconstitution at room temperature and injected into the system with a JASCO 880-PU intelligent pump (Tokyo, Japan) equipped with a multiwavelength detector and a flow-through Raytest-Gabi γ-detector. CC 250/4.6 Nucleosil 120-5 C-18 column from Teknokroma was used. 0.1% trifluoroacetic acid/water (Solvent A) and 0.1% trifluoroacetic acid/acetonitrile (Solvent B) were used as a mobile phase in the following gradient: 0 min 95% A (5% B), 5 min 95% A (5% B), 25 min 0% A (100% B), 30 min 0% A (100% B), flow = 1 ml/min, λ = 280 nm **Fig. 1.**

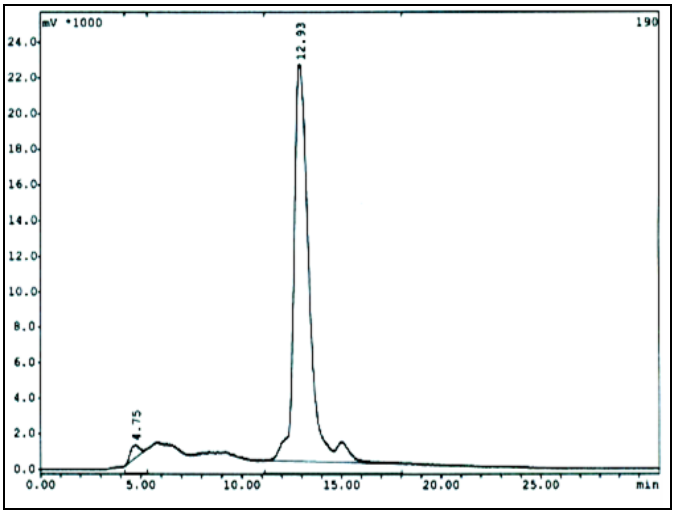

FIG. 1: RADIO-HPLC CHROMATOGRAM

Radio-HPLC profile of ^{99m}Tc - Ofloxacin radiocomplex has been obtained 1 h post reconstitution. ^{99m}TcO4 and ^{99m}Tc-Ofloxacin species were readily identified by Radio-HPLC. The retention times of ^{99m}TcO4 and ^{99m}Tc-Ofloxacin radiotracer were approximately 4.75 and 13 min respectively.

Radioisotope analysis: The rats were placed in the restrainer apparatus and the 37 MBq (1 mCi) ^{99m}Tc-Ofloxacin was injected intravenously by contra lateral tail vein in all studies. The animals were returned back to their cages and kept individually. Radioisotope analysis was performed 1 h post injection. Therefore, the animal were anesthetised by diethyl ether and placed on the board in the supine position with limbs spread out and fixed on the board with surgical tape. A single-headed camera (E-Cam, Siemens USA) employing a low-energy high resolution was used in all investigations. Acquisition parameters were as follow: matrix size 256×256, Zoom factor ×3, anterior and posterior views for 5 min and energy window 140 Kev and reconstitution method: filter back projection. Anterior and posterior static images was acquired using a large field of view gamma camera peaked 140Kev with a 15 % window and a low energy all-purpose collimator for 500 kilocounts per image. The gamma camera was positioned to image the affected foot and contralateral healthy side.

Two criteria were considered for interpretation of radioisotope imaging. First, the visual inspectation of radiotracer uptake at the affected foot to the contralateral healthy side was performed **Fig. 2**. Second, by using available commercial soft ware the activity at the affected foot to the contralateral healthy side was quantified. For this reason the region of interest (ROI) was generated on the affected foot as a target, and then second ROI was created on the contralateral healthy side as non target in interior view. The specific accumulation of radiotracer was calculated by dividing count per pixel in target to non target. The back ground subtraction was not used.

All images were observed and interpreted by three independent nuclear physicians and their final opinion was achieved by consensus. This study was double-blind and therefore, the observers were unaware about the nature of induced injuries on the rat's foot. The quantitative analysis has been performed after imaging assessment. The rats were sacrificed by diethyl ether and the organs of interest such as affected foot, contra lateral healthy side foot, kidneys, liver, stomach, spleen, intestine, bladder, heart, and lungs removed and weighted. The relative activity of each organ to the interest organs was calculated. The results have been obtained from this analysis, stated in **Table 1**.

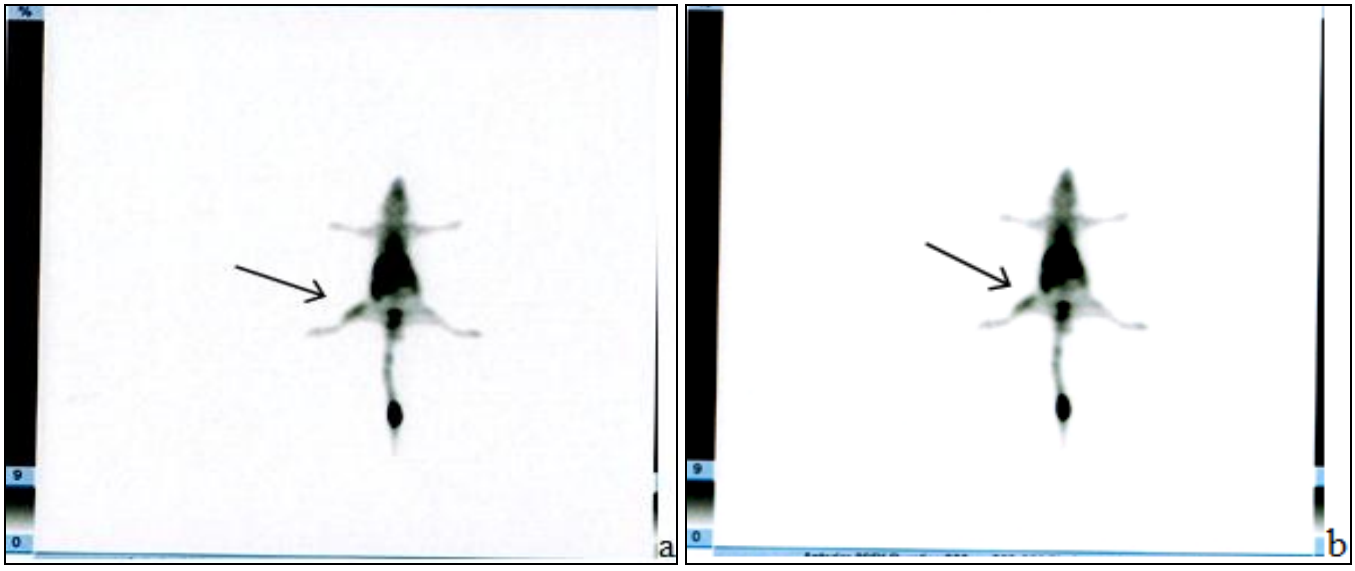

FIG. 2: SCINTIGRAPHY IMAGING

^{99m}Tc-Ofloxacin imaging has been undertaken 1h after 37 MBq (1 mCi) radiotracer injected by contra lateral tail vein. The anterior view images demonstrated lesions a: infection induced by *Staphylococcus aureus* b: sterile inflammation induced by Carrageenan assay.

TABLE 1: RELATIVE BIODISTRIBUTION OF ^{99M}TC- OFLOXACIN IN RAT

Organ / Relative uptake	Af	Uf	Li	In	Ki	St	Lu	Sp	Bl	He
Infection	7.71 ±3.35	4.62 ±1.67	36.25 ±18.21	16.93 ±3.6	12.73 ±6.59	3.61 ±0.9	3.02 ±0.97	1.02 ±0.24	12.7 ±8.3	1.95± 0.85
Inflammation	6.15 ±2.4	4.62 ±1.67	40.38 ±16.3	20.92 ±9.2	12.89 ±6.4	3.61 ±0.9	3.02 ±0.97	1.57 ±0.41	1.88 ±0.97	2.02± 1.2

Quantitative analysis has been performed after radioisotope imaging. Therefore, the rats were killed by diethyl ether and organ of interest like (Af: affected foot, Uf: unaffected foot, Li: liver, In: intestine, Ki: Kidneys, St: stomach, Lu: Lungs, Sp: Spleen, Bl: bladder and He: heart). The relative activity of each organ to the interest organs was calculated for different animals groups. The data in top row belong to the radiotracer biodistribution in the rats (n= 10) infected with S aureus. The data in bottom row belong to the radiotracer biodistribution in the rats (n = 10) with sterile inflammation lesion, which were induced by Carrageenan assay.

Statistic analysis: The calculations of means and standard deviations were made on Microsoft Excel. The data were shown as the mean ± SD. Repeated measure design analysis of variance followed by Tukey test was used to assess the difference between the radiotracer uptake at the infection and sterile inflammation lesions. Statistical significance was considered at $p < 0.05$.

RESULTS: Free $^{99m}TcO_4$ and $^{99m}TcO_2$ are two main radiochemical impurities produced during the labeling of ofloxacin cold kit with ^{99m}Tc radioisotope. The $^{99m}TcO_2$, $^{99m}TcO_4$ and radiotracer complex sample can be readily identified and quantified by ITLC analysis. But the $^{99m}TcO_2$ impurity could not be detected and determined by Radio-HPLC study. ^{99m} Tc-Ofloxacin radiotracer and $^{99m}TcO_4$ were readily identified by Radio-HPLC analysis. The potential capability of Radio-HPLC assay is identified and measured the proportion of $^{99m}TcO_4$ and radiocomplex. The TLC analysis indicated that the yield of (n= 10) radiocomplex, $^{99m}TcO_4$ and $^{99m}TcO_2$ were 92.55±2.65, 4.8± 1.32 and 2.65± 0.75 respectively. The radio-HPLC demonstrated that the yield of radiotracer complex and $^{99m}TcO_4$ were 98.14± 0.45 and 1.86± 0.15 respectively. The retention times of $^{99m}TcO_4$ and ^{99m}Tc-Ofloxacin complex were approximately 4.75 and 13 min. It revealed this matter that the labeling reaction was leaded to a single ^{99m}Tc-Ofloxacin radioconjugate.

The radiocomplex could be successfully prepared during labeling procedure. Radionuclide studies have been undertaken 48h post inoculation of bacteria samples. These investigations have been performed 2 h after aseptic inflammation foci induced by Carrageenan assay. All injuries were created in the right foot of animals in order to exclude any misinterpretation of radioisotope scintigraphy imaging. The visual inspection of images indicated that all affected feet were detected and identified by scintigraphy imaging. The quality of images was suitable in each subject and did not change over time. This matter revealed that the scintigraphy imaging with ^{99m}Tc-Ofloxacin is high sensitive to discriminate the lesions. But it was not specific to distinguish infection from sterile inflammation lesions. Semiquantitative and quantitative investigations have been performed in order to provide the further information about the accumulation and biodistribution of radiocomplex at the affected feet and the other organs in the rats. The target to non target ratios at the septic (n=10) and aseptic (n=10) lesions were 1.6± 0.12 and 1.3± 0.05 respectively.

The statistical significant difference in radiotracer uptake has not been observed between the infection and sterile inflammation lesions. Therefore, visual inspection of images could not discriminate septic from aseptic foci in this study.

Quantitative analysis has been performed in order to assess the biodistribution of radioconjugate at the different parts of rats. The ratios of target to non target were 1.64± 0.19 and 1.47± 0.15 at the infection and sterile inflammation lesions in quantitative study. It confirmed this matter that there is no significant difference in accumulation of radiotracer at the induced lesions. This achievement was very consistent to the outcome has been obtained by semiquantitative analysis. As it is shown in **Table 1**, the pathologic condition induced septic or aseptic inflammation could not lead to a considerable difference in biodistribution

of radiotracer in the interested organs (including affected foot, contra lateral healthy foot, liver, intestine, stomach, bladder, lungs, kidneys, heart and spleen) except bladder and lungs. The highest radioactivity was measured in liver followed by intestine and kidney. It indicated that the liver was the main organ for the metabolism of radiotracer. According to the results have been obtained from radioisotope analysis, the sensitivity and specificity of imaging with ^{99m}Tc-Ofloxacin radiocomplex were 100 and 50% to distinguish infection from sterile inflammation lesions.

DISCUSSION: The most challenging and critical stage in the management of patient is the diagnosis of disease with high accuracy. The identification of infection foci is still important for adequate treatment of patients. Available techniques are not sufficient to achieve this objective. The lack of substantial anatomical changes cannot be identified as early phase of development of disorder by plain radiography, CT scan and MRI. For this reason there is highly a demand for development method to distinguish infection foci. Radioisotope imaging is generally used in the condition of fever of unknown origin or suspected bacterial infection to help detection and to indicate the need for antimicrobial treatment. The early visualization of infection from sterile inflammation is one the most common problems in nuclear medicine. Therefore, several radiopharmaceutical agents have been examined to find the solution of this dilemma. Gallium 67 (^{67}Ga) is the most primitive radiotracer for scintigraphy imaging in this regard. But it is suffered from the following disadvantage points. It has long physical half-live decay, multiple gamma ionizing radiations causing high radiation absorbed doses and is not available as generator.

In addition to the above mentioned factors, ^{67}Ga is high sensitive for both infection and non-infectious inflammation and is the product of cyclotron and relative high expensive [13]. Bone imaging with ^{99m}Tc-MDP is widely used in nuclear medicine in this regard. In spite of high sensitivity of bone scan, it is not selective for infection lesion. The radiotracer uptake may be increased non-specifically for the presence of inflammation [14]. Labeled leukocyte with Indium 111(^{111}In) or ^{99m}Tc radioisotope is recommended as gold standard to visualize infection foci. It is necessary to take blood from patient, separate leukocyte and label with radionuclide. The labeled leukocyte must be reinjected to the patient. The labeling procedure is time-consuming and has potential inherent risk of contamination or transmission of blood-borne pathogens to patient or technician. This technique requires specialize facilities. In addition to the aforementioned factors, labelled leukocyte radiotracer cannot be used in neutropenic subjects [15, 16]. The broad spectrum antibiotic agents are readily taken and metabolized by microorganisms. These molecules can be accumulated by different mechanisms at the site of infection site. Therefore, the tiny amount of broad spectrum antibiotics has been evaluated as an infection-seeking imaging agent [17, 18].

The majority of the various antibiotic agents that have been investigated in this regard are those of the quinolones, second and third generation of cephalosporin agents. According to the literature, labeled antibiotic agents could demonstrate acceptable and promising sensitivity in detecting a wide variety of septic lesions [19-24]. Ofloxacin is a synthetic fluoroquinolones derivative with potent bactericidal activity against a wide spectrum of gram-negative and gram-positive bacteria [25, 26]. The new developed ^{99m}Tc-Ofloxacin combines the advantages of ^{99m}Tc and broad spectrum microorganisms localizing capability by bonding and inhibition of DNA gyrase. The therapeutic characteristics of ofloxacin have been used for diagnostic tool in order to tag the infection foci. The dose of ofloxacin versus to pharmacologic dose for eradication of infection is too low. The role of ofloxacin is a carrier for ^{99m}Tc radionuclide in order to transfer radiotracer to the target. Therefore, all induced infection lesions by *S. aureus* were identified and visualized by radioisotope imaging in this approach.

Carrageenan is a natural polysaccharide obtained from edible red seaweeds. Carrageenan assay is routinely used to assess anti inflammatory activity any compounds in an experimental animal model without any injury or damage to the inflamed tissue [27, 28]. Several mechanisms have been suggested for creation sterile inflammation following the injection of Carrageenan. Variety inflammatory mediators such as histamine, serotonin and bradykinin are released and detectable in the early

phase of sterile inflammation induced by Carrageenan. Prostaglandins are involved and caused the increased vascular permeability at the site of inflammation sites. The increased levels of other inflammatory mediators like Tumor Necrozing Factor (TNF), Interleukin 1 (IL-1) and IL-6 are responsible for local and systemic inflammation. In addition to the above mentioned factor, local neutrophil infiltration and activation are also accompanied to the inflammatory response by producing among other mediators [29-30].

The exact mechanism of accumulation of ^{99m}Tc-Ofloxacin radiotracer is not completely known at the inflammation foci. The following assumptions can be considered for the radiotracer uptake at the inflamed tissue. The radiotracer could be transferred at the inflamed area for the local congestion and increased vascular permeability produced after administration of Carrageenan solution. Fluoroquinolone agents could be taken up by neutrophil and activated macrophage [31].

Finally, non-specific bonding of radioconjugate to the other receptors may be considered. Therefore, the radiotracer could be delivered at the inflamed region at the sufficient concentration to tag and visualized affected area. For this reason all sterile inflammation lesions were identified by scintigraphy imaging studies. Carrageenan test can be considered as an ideal investigational assay in nuclear medicine in order to assess any radiopharmaceutical agents which are introduced as an infection-seeking agent in the pre clinical phase of analysis.

CONCLUSION: The outcome of this approach indicated that radioisopte imaging is too sensitive to subtle changes in physiological processes. In spite of high sensitivity of imaging with new developed ^{99m}Tc-Ofloxacin radiotracer, it was exhibited low specificity for differentiation between infection and sterile inflammation foci. It is mandatory to consider the other modalities for intelligent interpretation of ^{99m}Tc-Ofloxacin scintigraphy images.

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CONFLICT OF INTEREST: This approach is part of Pharm-D thesis of Maryam Saadi. Authors have no relevant financial interests related to the material in this manuscript. They also have no conflict of interests to declare.

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